GENETICS

Mycoplasma-Mediated Uptake of the Exogenous Human BRCA1 Gene by Hatching Blastocysts¹

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Purpose: Biological vectors for cell transfection are mainly viral in origin, with inherent shortcomings. Mycoplasmas are ubiquitous organisms that traverse cells easily. The objective was to determine if *Ureaplasma urealyticum* (T-mycoplasma) would vector exogenous BRCA1 DNA into blastocysts.

Methods: Hatching mouse blastocysts ($N = 70$) were incubated in the presence of either viable or dead *Ureaplasma urealyticum* at 37°C for 1 hr. The blastocysts were exposed to human BRCA1 DNA lacking homology in the mouse genome for 2 hr, followed by DNase-I treatment and wash. Polymerase chain reaction and agarose gel electrophoresis analysis of amplified products were performed.

Results: The BRCA1 gene was detected in the blastocysts only when viable *Ureaplasma* was present. PCR analyses of control *Ureaplasma* and untreated blastocysts were negative.

Conclusion: Viable *Ureaplasma* organisms were shown to mediate the uptake of DNA fragments into blastocysts, resulting in transgenic mouse blastocysts with a normal human BRCA1 exon 11 gene.

KEY WORDS: breast cancer; mycoplasma; *Ureaplasma urealyticum*; foreign DNA; gene transfer; transgenic.

INTRODUCTION

The uptake of exogenous or foreign DNA fragments by the cells of blastocysts has been reported (1–4).

The DNA fragments used in the uptake studies were derived from extracted cells (1) and from human papillomavirus (2–4). Gene transfer into the blastocyst has also been accomplished in bovine species using replication-defective retroviral vectors (5). However, the transfer of important genes such as the normal tumor suppressor gene for breast cancer, BRCA1, into blastocysts has not been reported.

In this laboratory, it was found that the exposure of blastocysts to BRCA1 DNA fragments in culture was not sufficient to yield an efficient transfection of this exogenous DNA into the cells of the blastocyst. It was thought that either there was no protection from membrane DNase and intracellular methylase that modified the exogenous DNA (6) or there was no transport across the cell membrane without the aid of carriers such as liposomes (7). Since wall-free mycoplasma microorganisms are well known for their capacity to cross cell membranes (8,9), it was hypothesized that these organisms would vector the BRCA1 gene into blastocysts using the “coat-tail” effect.

Hence, the objective was to determine if *Ureaplasma urealyticum* (T-mycoplasma) would facilitate the uptake of normal BRCA1 DNA fragments into hatching blastocysts. The results of this study would help expand our knowledge of the role of mycoplasmas in genetic transformation and open up the possibility of using inactivated mycoplasmas to mediate gene transfer.

MATERIALS AND METHODS

Preparation of Blastocysts

Mature female mice were stimulated with 5 IU pregnant mare serum gonadotropin (PMSG; Sigma Chemi-

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cal Co, St. Louis, MO) followed by 5 IU human chorionic gonadotropin (hCG; Sigma Chemical Co.) and mated with a proven male 2 days after hCG treatment. Pronuclear-stage embryos were flushed from the oviducts 20 hr after mating. The pronuclear-stage embryos were incubated in supplemented HTF (synthetic human tubal fluid) medium containing 4 mg/ml bovine serum albumin (BSA; Sigma Chemical Co.) until they reached the hatching blastocyst stage after about 100 hr of incubation. The stage of embryonic development was visually confirmed using light microscopy. The hatching blastocyst stage was used here, as this was one of the most vulnerable time periods for the embryo that did not have the protection of the zona pellucida. A few of the mouse embryos that failed to reach the blastocyst stage, characterized by dark cytoplasm with fragmented blastomeres, were not used.

Hatching-stage mouse blastocysts ($n = 70$) were divided and incubated in culture dishes under oil in the presence of either viable *Ureaplasma urealyticum* (reconstituted T-mycoplasma control organisms; Cat. No. D301-004; Irvine Scientific, Santa Ana, CA) or dead *Ureaplasma urealyticum* (preheated at 100°C for 30 min and cooled to 37°C). Untreated blastocysts in the supplemented HTF medium served as a negative control. Incubation conditions were 37°C for 1 hr in a 5% CO₂ in air-mixture.

After incubation, 10 µl of transfection solution containing human BRCA1 exon 11 DNA fragments was added to each culture dish of either viable *Ureaplasma*-treated blastocysts, nonviable *Ureaplasma*-treated blastocysts, or viable *Ureaplasma* organisms without blastocysts (second negative control). Incubation was carried out for 2 hr in a 5% CO₂-in-moist air mixture. The transfection solution was prepared by diluting 1:20 the PCR-amplified product of a previous PCR run with supplemented HTF medium for a final concentration of about 0.01 µg DNA/µl. The previous PCR run based on a single source of human donor sperm cells targeted the BRCA1 exon 11 gene, base positions 36,863 to 37,286 (10), generating a 434-base pair product and previously verified using 2% agarose gel electrophoresis and ethidium bromide staining techniques.

After incubation, each group of blastocysts or control was treated with deoxyribonuclease I solution (DNase I; Sigma Chemical Co.) for 10 min at 37°C to remove extracellular DNA fragments and washed through three changes of HTF medium. The blastocysts were pipetted into PCR reaction tubes ($n = 7$ per tube). Aliquots (10 µl each) of the control *Ureaplasma* solution or the transfection solution were also placed

into reaction tubes. All reaction tubes with specimens were frozen until used.

Polymerase Chain Reaction (PCR) Protocol

The reaction tubes were removed from the freezer and mineral oil (50 µl per tube) was added to the tubes to prevent evaporation. The reaction tubes were heated at 100°C for 20 min to inactivate DNase I and hot-start was performed for 7 min at 95°C. PCR reaction cocktail mixture was added to each tube, and PCR performed in a Perkin Elmer Cetus Thermal Cycler (Norwalk, CT). The cocktail mixture consisted of 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 4.0 mM MgCl₂, a 200 µM concentration of each dNTP, 100 µg/ml gelatin, a 1 µM concentration of each pair of primers targeting the BRCA1 gene (Bio-Synthesis Inc., Lewisville, TX), and 1.3 U Taq polymerase enzyme (AmpliTaq; Perkin Elmer Cetus, Norwalk, CT). As mentioned above, the PCR amplified a 434-base pair (bp) product. The cycling parameters were set for 32 step-cycles with 40 sec of denaturation at 94°C, 40 sec of annealing at 60°C, and 40 sec of extension at 72°C, followed by a final extension at 72°C for 7 min. Each amplified product was pipetted out, mixed with 5 × loading buffer, and loaded into wells, and 2% agarose gel electrophoresis performed. For each agarose run, the *Hae*III digest of φX174 served as the marker. Amplified DNA bands were analyzed with ethidium bromide staining and visualized by means of ultraviolet illumination.

RESULTS

Exogenous DNA from the breast cancer gene, BRCA1, was transfected into hatching blastocysts when viable *Ureaplasma* organisms were present (Fig. 1). There was no uptake of exogenous DNA in blastocysts when the *Ureaplasma* organisms were nonviable. PCR analyses of viable *Ureaplasma* organisms without the blastocysts showed an absence of the human BRCA1 gene. Similarly, analysis of untreated mouse blastocysts was negative for the human BRCA1 gene. Residual culture media containing the BRCA1 DNA transfection medium recovered from both the viable and the nonviable *Ureaplasma*-treated blastocysts culture dishes were positive for the BRCA1 gene. The positive control based on human donor sperm was also positive for BRCA1.

Fig. 1. Analysis of PCR-amplified products for the human BRCA1 exon 11 gene (434 base pairs) in hatching mouse blastocysts after transfection in the presence or absence of *Ureaplasma urealyticum* (T-mycoplasma) using 2% agarose gel electrophoresis, followed by ethidium bromide staining and visualization in ultraviolet light. Lane M, the DNA marker from the *Hae*III digest of ϕ X174; Lane 1, the residual transfection medium used in the viable *Ureaplasma* group; Lane 2, blastocysts treated with viable *Ureaplasma* and exogenous BRCA1 fragments, followed by DNase I, wash, and enzyme heat inactivation; Lane 3, the residual transfection medium used in the non viable *Ureaplasma* group; Lane 4, blastocysts treated with nonviable *Ureaplasma* and exogenous BRCA1 fragments followed by DNase I, wash, and enzyme heat inactivation; Lane 5, the negative control of viable *Ureaplasma* treated with BRCA1 fragments; Lane 6, the positive control consisting of human sperm cell DNA.

DISCUSSION

The present study demonstrated an uptake of exogenous DNA by blastocysts that was mediated by viable *Ureaplasma urealyticum* (T-mycoplasma) organisms. In this case, the normal human BRCA1 exon 11 gene was taken into the hatching blastocysts. The mouse blastocyst was used as the target of transfection since the PCR primers, specific for the human BRCA1 gene, would not yield artifact products in this model. Similarly, the *Ureaplasma* organism did not produce artifact BRCA1 products. The data suggest the possibility of using inactivated mycoplasma to vector normal DNA genes into embryos. The data also serve to alert of the possibility of the uptake of viral or bacterial DNA into embryos mediated by mycoplasmas which may have undesirable consequences.

Exogenous DNA taken into the cells either remain as episomes or become randomly integrated into the host DNA, eventually disrupting normal cell function (11). It has been reported that 55% of patients with unexplained infertility are infected with *Ureaplasma urealyticum*, significantly above the control population (12). *Ureaplasma* or mycoplasma infection has been linked to miscarriages (13–15). It remains to be seen if infertility is a result of the uptake of exogenous deleterious DNA into the embryos mediated by the *Ureaplasma* organisms. This is in addition to the other documented effects of mycoplasmas on cells such as the release of nucleases (16), reactive oxygen mole-

cules (17), or superantigens which affect cellular replication (18). Nonetheless, the present results suggest the capacity of mycoplasmas to facilitate the uptake of exogenous DNA.

In a positive way, desirable DNA fragments such as the normal BRCA1 gene may be taken into cells through inactivated mycoplasmas for the purposes of gene therapy. Once the desired DNA is inside the target cell, common antibiotics can be used to eliminate the mycoplasma vectors, leaving the cell to incorporate the exogenous DNA. Note, however, that the mycoplasmas producing a “coat-tail” effect, may also be damaging when they facilitate the uptake of viral or bacterial DNA. This becomes relevant in assisted reproduction technology, where there are several opportunities for mycoplasmas to infect, for example, during intracytoplasmic sperm injection (ICSI), blastocyst culture, or the first trimester of pregnancy, when the prophylactic antibiotic doses prescribed earlier would be long gone.

The mechanism involved in the mycoplasma-mediated DNA uptake process remains unknown. Several possible mechanisms exist. The mycoplasma organism, along with the exogenous DNA, may be internalized into the blastocyst cell by endocytosis or the mycoplasmas may alter the permeability of the cell membrane during the cell invading phase. What is now known is that the uptake process is an active process requiring viable mycoplasma organisms. When the organisms were destroyed by heat, there was no evidence of uptake of the BRCA1 gene. Although it has been reported that mycoplasmas have the capacity to internalize nucleotides (19) into their wall-free structures, there was no evidence of the uptake of the 434-bp BRCA1 gene fragment into these organisms as shown by the control in this study.

Mycoplasmas are tiny polymorphic prokaryotic organisms (0.2–0.3 μ m) lacking a cell wall residing ubiquitously at the cell membrane level or internalized into the cell (8,9). These organisms have been implicated in pneumonia, postabortal fever, pelvic inflammatory disease, pyelonephritis (20), endometritis (21), urethritis (22), perinatal mortality, arthritis (23), cancer (24), spontaneous abortion (13–15), infertility (25), and interference with sperm production and function (26) and as a cofactor in HIV infection (27). Many species of mycoplasmas exist and five of these are found in the human genitourinary tract: *M. hominis*, *M. fermentans*, *M. primatum*, *M. genitalium*, and *U. ureaplasma* (28). While the technique of using inactivated adenovirus to transfer exogenous DNA to host cells has been reported (29–31), the use of other organ-

isms has not been given full consideration; hence, the present study on mycoplasma-mediated gene transfer. The next step would be to define methodology to inactivate the mycoplasmas while retaining the transfecting capacity. In addition to expanding our knowledge of mycoplasma-mediated genetic transformation of cells, the possible applications of inactive mycoplasma-mediated transgenic animal production invite exploration by other researchers.

In summary, *Ureaplasma* or T-mycoplasmas were shown to facilitate the entry of DNA fragments into hatching blastocysts. The study suggests that useful DNA fragments such as the normal *BRCA1* gene may be transfected into cells using mycoplasmas. On the other hand, the blastocyst may be vulnerable to the entry of undesirable DNA from eclectic sources when mycoplasmas are present. The exact mechanism remains unknown but it is an active process requiring viable transfecting organisms. More studies are needed to examine the role of mycoplasmas in genetic transformation and natural production of transgenic offsprings.

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